APC germline mutations in families with familial adenomatous polyposis

LILLIAN BARBOSA DE QUEIROZ ROSSANESE[1*], FERNANDO AUGUSTO DE LIMA MARSON[1,2*], JOSÉ DIRCEU RIBEIRO[2], CLAUDIO SADDY RODRIGUES COY[3] and CARMEN SILVIA BERTUZZO[1]

Departments of [1]Medical Genetics, [2]Pediatrics and [3]Surgery, Medical Science Faculty, University of Campinas, Cidade Universitária, Campinas, SP 13081-970, Brazil

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Abstract. Adenomatous polyposis coli (*APC*) germline mutations are responsible for the occurrence of familial adenomatous polyposis (FAP). Somatic mutations lead to malignant transformation of adenomas. In this context, considering the significance of *APC* germline mutations in FAP, we aimed to identify *APC* germline mutations. In the present study, 20 FAP patients were enrolled. The determination of *APC* germline mutations was performed using sequencing, and the mutations were compared with clinical markers (gender, age at diagnosis, smoking habits, TNM stage, Astler-Coller stage, degree of differentiation of adenocarcinoma). The data were compared using the SPSS program, with the Fisher's exact test and χ^2 test, considering α=0.05. According to the main results in our sample, 16 alleles with deleterious mutations (80% of the patients) were identified while 7 (35%) patients had no deleterious mutations. There was a predominance of nonsense (45% of the patients) and frameshift (20% of the patients) mutations. There was no statistical significance between the *APC* germline mutations identified and the clinical variables considered in our study. Only TNM stage was associated with the presence of deleterious mutations. Patients with deleterious mutations had an OR, 0.086 (IC=0.001-0.984); TNM stage I + II in comparison with III + IV, when compared with the patients with no deleterious mutations identified. In this context, as a conclusion, we demonstrated the molecular heterogeneity of *APC* germline mutations in FAP and the difficulty to perform molecular diagnostics in a Brazilian population, considering the admixed population analyzed.

Introduction

Colorectal cancer is common in Brazil. In the year 2012, 14,180 new cases of colon and rectum cancer were expected to occur in men and 15,960 in women. These values correspond to an estimated risk of 15 new cases per 100,000 men and 16 cases per 100,000 women (1).

Excluding non-melanoma skin tumors, colon and rectum cancer is the second most common cancer among men in Southeast Brazil (22/100,000) and third in South (18/100,000) and Midwest (14/100,000) Brazil (1). In North Brazil (4/100,000) this cancer ranks fourth; in Northeast Brazil (5/100,000), fifth. Among women, it is the second most common cancer in Southeast (23/100,000) and South Brazil (20/100,000), the third in Midwest (15/100,000) and Northeast Brazil (7/100,000), and sixth in the North (5/100,000) (1).

Familial adenomatous polyposis (FAP) is one of the most clearly defined and well understood inherited colorectal cancer syndrome. It is an autosomal dominant disorder that typically presents in the form of colorectal cancer in young adults secondary to extensive adenomatous polyposis present in the colon (2).

The adenomatous polyposis coli (*APC*) gene is on chromosome 5q21 and displays alternative splicing in multiple coding and noncoding regions of the DNA sequence, and the primary transcript has 15 exons. The *APC* gene has 8,532 base pairs corresponding to 2,844 amino acids, resulting in a 311.8-kDa protein. Exon 15 has the largest extension, making up more than three quarters of the coding region (3).

Approximately 737 *APC* gene mutations, including 332 germline and 402 somatic have been identified. *APC* germline mutations are responsible for the occurrence of FAP, and somatic mutations have been associated with malignant transformation of adenomas (4). Almost all mutations lead to truncation of the APC protein either by nonsense (30%) or by frameshift mutations (68%). The majority of mutations occur within the first half of the coding sequence. In an American study, in which 1,591 patients were studied, of the 431 pathogenic or likely pathogenic mutations, frameshift, nonsense,

Correspondence to: Dr Fernando Augusto De Lima Marson, Department of Medical Genetics, Medical Science Faculty, University of Campinas, Cidade Universitária, Tessália Vieira de Carmargo 126, Campinas, SP 13081-970, Brazil
E-mail: fernandolimamarson@hotmail.com

*Contributed equally

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splice sites and large deletion or duplication mutations represented 43, 42, 9 and 6% of cases, respectively (5).

APC germline mutations are predominate at the 5' end of the gene, while somatic mutations mainly occur in the region called the mutation cluster region (MCR) between codons 1,284 and 1,580 of the *APC* gene. In germline mutations, two hot spot codons have been identified; one at position 1,061 and the second at position 1,309. In somatic mutations, two hot spots seem to occur at position 1,309 and 1,450 (3).

Several studies have attempted to correlate specific *APC* mutations with clinical phenotypes. Mutations between codons 169 to 1,578 have been generally associated with the classic form of FAP. Mutations between codons 1,445 and 1,578 have been associated with desmoid tumors, whereas mutations between codons 279 to 1,309 have been correlated with the development of duodenal polyposis (6).

Based on the findings in the literature, the objective of the present study was to detect *APC* germline mutations that affect families followed up at the Oncology Clinic of the University of Campinas (Unicamp) and to compare the identified mutations with clinical variables.

Materials and methods

We recruited 20 nonrelative patients at the Oncology Service in the 'Gastrocentro' of the Faculty of Medical Sciences of Unicamp. The present study included families that had two or more successive generations affected by FAP (>100 polyps); no polyposis colorectal cancer was present. The project was approved by the university ethics committee (#874/2008). All patients and/or their guardians signed an informed consent form.

Clinical variables. The clinical variables analyzed in our samples included gender (male/female), age at diagnosis (≤41 or >41 years), smoking habits (passive smoking, smoker, nonsmoker), TNM stage (I + II vs. III + IV), Astler-Coller stage (B1 + B2 vs. C1 + C2), degree of differentiation of adenocarcinoma (moderately differentiated, poorly differentiated, well-differentiated).

All of the variables were evaluated by medical specialists including special considerations to TNM stage (tumor, lymph node, metastasis), Astler-Coller stage and degree of differentiation of the adenocarcinoma and were evaluated taking into account previously literature (6-10).

DNA extraction. Genomic DNA was obtained by direct extraction from lymphocytes of peripheral blood according to standard procedures (11). DNA samples were quantified using the NanoVue® v1.7.2 spectrophotometer (GE Healthcare, Chicago, IL, USA). For all analyses performed, 50 ng/μl was used to improve the polymerase chain reaction (PCR) technique.

DNA sequencing and analysis. To identify *APC* mutations, DNA fragments containing the entire coding region and intron-exon boundaries of the *APC* gene were amplified, using PCR conditions as published by Miyoshi *et al* (12), Nagase *et al* (13) and Gómez-Fernández *et al* (14), with primers as listed in Table I. The precise gradients for temperature and buffers providing the optimal temperature for each fragment were determined experimentally. The PCR products

Table I. Description of the oligonucleotides used for the analysis of the *APC* gene.

Nucleotide nomenclature	Sequences
APC_EX1_F	5'-AACCTTATAggTCCAAgggTAg-3'
APC_EX1_R	5'-ACCTCAAgTTTACAAgAgggAA-3'
APC_EX2_F	5'-AAATACAgAATCATgTCTTgAAgT-3'
APC_EX2_R	5'-ACACCTAAAgATgACAATTTgAg-3'
APC_EX3_F	5'-gACCCAAgTggACTTTTCAgg-3'
APC_EX3_R	5'-ACAATAAACTggAgTACACAAgg-3'
APC_EX4_F	5'-gAgAAgTTTgCAATAACAACTgATg-3'
APC_EX4_R	5'-TTATCCTgAATTTTAATggATTACCT-3'
APC_EX5_F	5'-AACCTCACTCTAACTggACCAA-3'
APC_EX5_R	5'-AACAgAgCTgTAATTCATTTTATTCC-3'
APC_EX6_F	5'-ggTAgCCATAgTATgATTATTTCT-3'
APC_EX6_R	5'-CTACCTATTTTTATACCCACAAAC-3'
APC_EX7_F	5'-AAgAAAgCCTACACCATTTTTgC-3'
APC_EX7_R	5'-gATCATTCTTAgAACCATCTTgC-3'
APC_EX8_F	5'-gACACTTCATTTggAgTACCTTAACA-3'
APC_EX8_R	5'-ggCATTAgTgACCAgggTTT-3'
APC_EX9_F	5'-AgTCgTAATTTTgTTTCTAAACTC-3'
APC_EX9_R	5'-TTTgAAACATgCACTACgAT-3'
APC_EX10_F	5'-TTgCTCTTCAAATAACAAAgCAT-3'
APC_EX10_R	5'-TCCACCAgTAATTgTCTATgTCA-3'
APC_EX11_F	5'-gATgATTgTCTTTTTCCTCTTgC-3'
APC_EX11_R	5'-CTgAgCTATCTTAAgAAATACATg-3'
APC_EX12_F	5'-TgACAAAggAAgAACAgATAgCA-3'
APC_EX12_R	5'-gCAgTgAgCTgAgATTgCAC-3'
APC_EX13_F	5'-TTTCTATTCTTACTgCTAgCATT-3'
APC_EX13_R	5'-ATACACAggTAAgAAATTAggA-3'
APC_EX14_F	5'-AgggACgggCAATAggATAg-3'
APC_EX14_R	5'-ggTCTTTTTgAgAgTATgAATTCTg-3'
APC_EX15A_F	5'-TTgTTACTgCATACACATTg-3'
APC_EX15A_R	5'-CAAATATggTgAAAggACA-3'
APC_EX15B_F	5'-CCCTAgAAgCAgAATTAg-3'
APC_EX15B_R	5'-TTCTTCTAAgTgCATTTC-3'
APC_EX15C_F	5'-CATggAAgAAgTgTCAgC-3'
APC_EX15C_R	5'-TTCTATTATgTgTTTgggTC-3'
APC_EX15D_F	5'-CACAgAATgAAAgATggg-3'
APC_EX15D_R	5'-gAAggTgTggACgTATTC-3'
APC_EX15E_F	5'-gAAACgTCATgTggATCAgC-3'
APC_EX15E_R	5'-TggCAATCgAACgACTCTC-3'
APC_EX15F_F	5'-CCTAgAACCAAATCCAgCAgAC-3'
APC_EX15F_R	5'-gTTggCATggCAgAAATAATAC-3'
APC_EX15G_F	5'-AgATgCTTgCTggACCTg-3'
APC_EX15G_R	5'-TTgCCACggAAAgTACTC-3'
APC_EX15H_F	5'-TCTTgCAgAATgCATTAATT-3'

APC, adenomatous polyposis coli.

Table II. The familial adenomatous polyposis patients relating to gender, race, age at diagnosis, staging (TNM and Astler-Coller), smoking habit, degree of differentiation of adenocarcinoma and *APC* gene genotype.

Patient	Gender	Race	Age at diagnosis (years)	Staging		Smoking habit	Degree of differentiation in adenocarcinoma	Genotype	Mutation deleterious/not deleterious	Extra-colonic manifestations
				TNM	Astler-Coller					
1	F	C	37	IV	C1	NS	MD	Glu1309X	D	Small intestine and duodenal polyps
2	F	C	47	III	B2	S	MD	Ser 932 X	D	Duodenal polyps
3	F	NC	31	IV	C2	NS	MD	Tyr935X	D	Gastric polyps
4	F	C	40	II	C2	S	MD	Arg657Arg[a]/c.3927_3931 delAAAGA	ND/D	Small intestine polyps
5	F	C	45	IV	B2	S	MD	-	-	-
6	M	C	41	II	B1	S	MD	Ile606Ile[a]	ND	-
7	M	C	36	I	C2	NS	PD	Gln1291X	D	Small intestine and duodenal polyps
8	F	C	29	III	C2	NS	MD	Gly2502Ser	D	Gastric polyps
9	M	C	47	II	C2	S	MD	Glu1317Gln	D	Osteoma jaw
10	F	C	41	III	B2	NS	MD	Leu629X	D	Duodenal polyps
11	F	C	52	II	B2	NS	PD	Asn1037Asn[a]/Tyr935X	ND/D	-
12	F	NC	40	I	C2	NS	WD	Thr934Thr[a]	ND	-
13	F	C	44	III	C1	NS	MD	c.3183_3186delACAA	D	Small intestine polyps
14	M	C	36	IV	B2	NS	MD	c.3183_3187delACAAA	D	Small intestine polyps
15	F	C	55	IV	C2	S	MD	Arg 876X	D	-
16	M	C	49	II	B1	PS	MD	Lys939Lys[a]/Tyr951Tyr[a]	ND/ND	Gastric polyps
17	F	C	50	II	NR	NS	WD	Glu892X	D	-
18	F	C	41	III	C2	NS	PD	Gly974Gly[a]/ c.3927_3931delAAAGA	ND/D	-
19	F	C	47	III	C2	NS	MD	Lys1454Glu	D	-
20	M	C	49	IV	C2	PS	MD	Leu1564X	D	Duodenal polyps

[a]Neutral mutation. *APC*, adenomatous polyposis coli; F, female; M, male; C, Caucasian, NC, not Caucasian; NS, non-smoker; PS, possible smoker; S, smoker; MD, moderately differentiated; PD, poorly differentiated; WD, well-differentiated; D, deleterious mutations; ND, no deleterious mutations.

Table III. Association of clinical variables of colorectal cancer according to TNM stage.

Variable	TNM stage		Total	P-value	OR	CI (5-95%)
	I and II n (%)	III and IV n (%)				
Gender						
Female	4 (28.6)	10 (71.4)	14	0.274	0.219	0.014-2.242
Male	4 (66.7)	2 (33.3)	6		1	-
Race						
Caucasian	7 (38.9)	11 (61.1)	18	1	0.615	0.007-57.02
Not Caucasian	1 (50)	1 (50)	2		1	-
Presence of deleterious allele						
Presence	5 (31.3)	11 (68.8)	16	**0.048**	**0.086**	**0.001-0.984**
Absence	6 (85.7)	1 (14.3)	7		**1**	-
Degree of differentiation of adenocarcinoma						
WD	2 (100)	-	2	-	-	-
MD	4 (26.7)	11 (73.3)	15	0.116	0.205	0.001-1.457
PD	2 (66.7)	1 (33.3)	3	0.688	3.424	0.149-235.6
Age at diagnosis (years)						
≤41	4 (40)	6 (60)	10	1	1	0.119-8.417
>41	4 (40)	6 (60)	10		1	-
Astler-Coller stage						
B1 and B2	3 (42.9)	4 (57.1)	7	1	1.468	0.142-14.66
C1 and C2	4 (33.3)	8 (66.7)	12		1	-
Smoking habit						
NS	4 (33.3)	8 (66.7)	12	0.777	0.518	0.056-4.451
S and PS	4 (50)	4 (50)	8		1	-

TNM system: T, describes how far the main (primary) tumor has grown into the wall of the intestine and whether it has grown into nearby areas; N, describes the extent of spread to nearby (regional) lymph nodes; M, indicates whether the cancer has spread (metastasized) to other organs of the body. Astler-Coller classification: A, tumor limited to the mucosa, carcinoma *in situ*; B1, tumor grows through muscularis mucosa but not through muscularis propria; B2, tumor grows beyond muscularis propria; C1, stage B1 with regional lymph node metastases; C2, stage B2 with regional lymph node metastases; D, distant metastases. Statistical analysis conducted by Fisher's exact test and χ^2 test. In bold print, P-values <0.05. OR, odds ratio; CI, confidence interval; NS, non-smoker; PS, possible smoker; S, smoker; MD, moderately differentiated; PD, poorly differentiated; WD, well-differentiated.

indicating heterozygosity were sequenced using the Applied Biosystems (ABI) Prism BigDye Terminator v3.1 cycle sequencing kit and ABI 3500XL DNA sequencer (PE Applied Biosystems, Foster City, CA, USA), using identical conditions as previously published (12-14). The DNA sequence was analyzed using GeneMapper software (Applied Biosystems) or Fragment Profiler (GE Healthcare Biosciences, Piscataway, NJ, USA).

Statistical analysis. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) v.17.0 from SPSS, Inc., Chicago, IL, USA (http:// www.spss.com) by Fisher's exact test and χ^2 test, considering α=0.05. To improve the data presentation, the odds ratio was calculated to variables to demonstrate the association between the clinical variables, TNM and *APC* germline mutation identified.

Results

In the descriptive analysis, the average age at diagnosis of the patients was 42.85 years (±6.892), and the age range was from 29 to 55 years. Of the 20 patients, 18 (90%) were Caucasian and 2 (10%) were not Caucasian; 14 (70%) were females and 6 (30%) were males. The clinical data are summarized in Table II. The frequency of cases for each stage according to the TNM system was 2 (10%), 6 (30%), 6 (30%) and 6 (30%), respectively, for stage I, II, III and IV. The frequency of cases for each stage according to the Astler-Coller system was 2 (10.5%), 5 (26.4%), 2 (10.5%) and 10 (52.6%), respectively, for stages B1, B2, C1 and C2. A smoking habit was observed in 8 patients, 2 (10%) were occasional smokers and 6 (30%) were smokers; the remaining patients (60%) were non-smokers. Two patients (10%) had well-differentiated adenocarcinoma,

Table IV. Association of colorectal cancer clinical variables according to Astler-Coller stage.

Variable	Astler-Coller B1 and B2 (no.)	Astler-Coller C1 and C2 (no.)	Total	P-value	OR	CI (5-95%)
Gender						
Female	4	9	13	0.617	0.465	0.04-5.09
Male	3	3	6		1	-
Race						
Caucasian	7	10	17	0.509	-	-
Not Caucasian	0	2	2		-	-
Presence of deleterious allele						
Presence	5	10	15	**0.376**	**0.393**	**0.04-3.36**
Absence	4	3	7		**1**	-
Degree of differentiation of adenocarcinoma						
WD	0	1	1	-	-	-
MD	6	9	15	0.607	1.933	0.12-122.2
PD	1	2	3	0.841	0.841	0.01-19.64
Age at diagnosis (years)						
≤41	3	7	10	0.650	0.554	0.05-5.03
>41	4	5	9		1	-
TNM stage						
I and II	3	4	7	1	0.554	0.05-5.03
III and IV	4	8	12		1	-
Smoking habit						
NS	3	8	11	0.377	0.396	0.04-3.67
S and PS	4	4	8		1	-

Astler-Coller classification: A, tumor limited to mucosa, carcinoma *in situ*; B1, tumor grows through muscularis mucosa but not through muscularis propria; B2, tumor grows beyond muscularis propria; C1, stage B1 with regional lymph node metastases; C2, stage B2 with regional lymph node metastases; D, distant metastases. TNM system: T, describes how far the main (primary) tumor has grown into the wall of the intestine and whether it has grown into nearby areas; N, describes the extent of spread to nearby (regional) lymph nodes; M, indicates whether the cancer has spread (metastasized) to other organs of the body. Statistical analysis conducted by Fisher's exact test and χ^2 test. In bold print, P-values <0.05. OR, odds ratio; CI, confidencial interval; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; NS, non-smoker; S, smoker; PS, possible smoker.

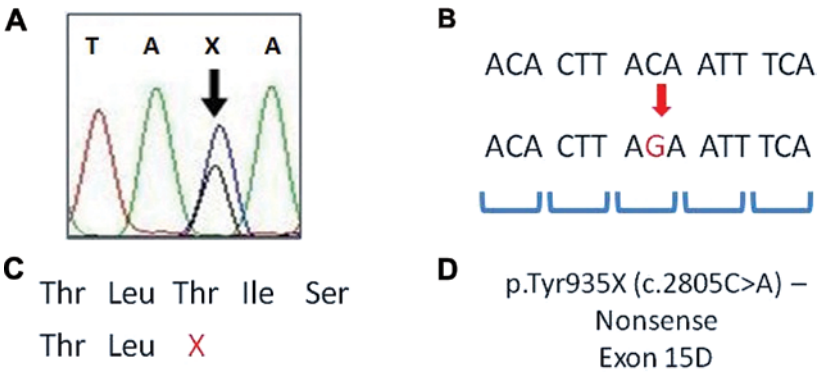

Figure 1. Representation of adenomatous polyposis coli (APC) mutation analysis. (A) DNA sequencing electropherogram of p.Tyr935X of *APC* gene; X, stop codon mutation. (B) DNA sequence with an alteration (red sequence - change of C to G, stop codon mutation); codons are shown in blue. (C) Amino acid sequence of protein and the stop codon in the APC partial amino acid sequence; X, stop codon in Thr amino acid. (D) Mutation description - identification, type of mutation, localization of mutation in *APC* gene. Thr, threonine; Tyr, tyrosine; A, adenine, T, thymine; C, cytosine; G, guanine. Fig. 1 was adapted from previous studies (15-17).

15 (75%) had moderately differentiated and 3 (15%) had poorly differentiated adenocarcinoma.

For the determined mutant alleles, 16 (40%) were deleterious and 7 (17.5%) were not deleterious. Associations were

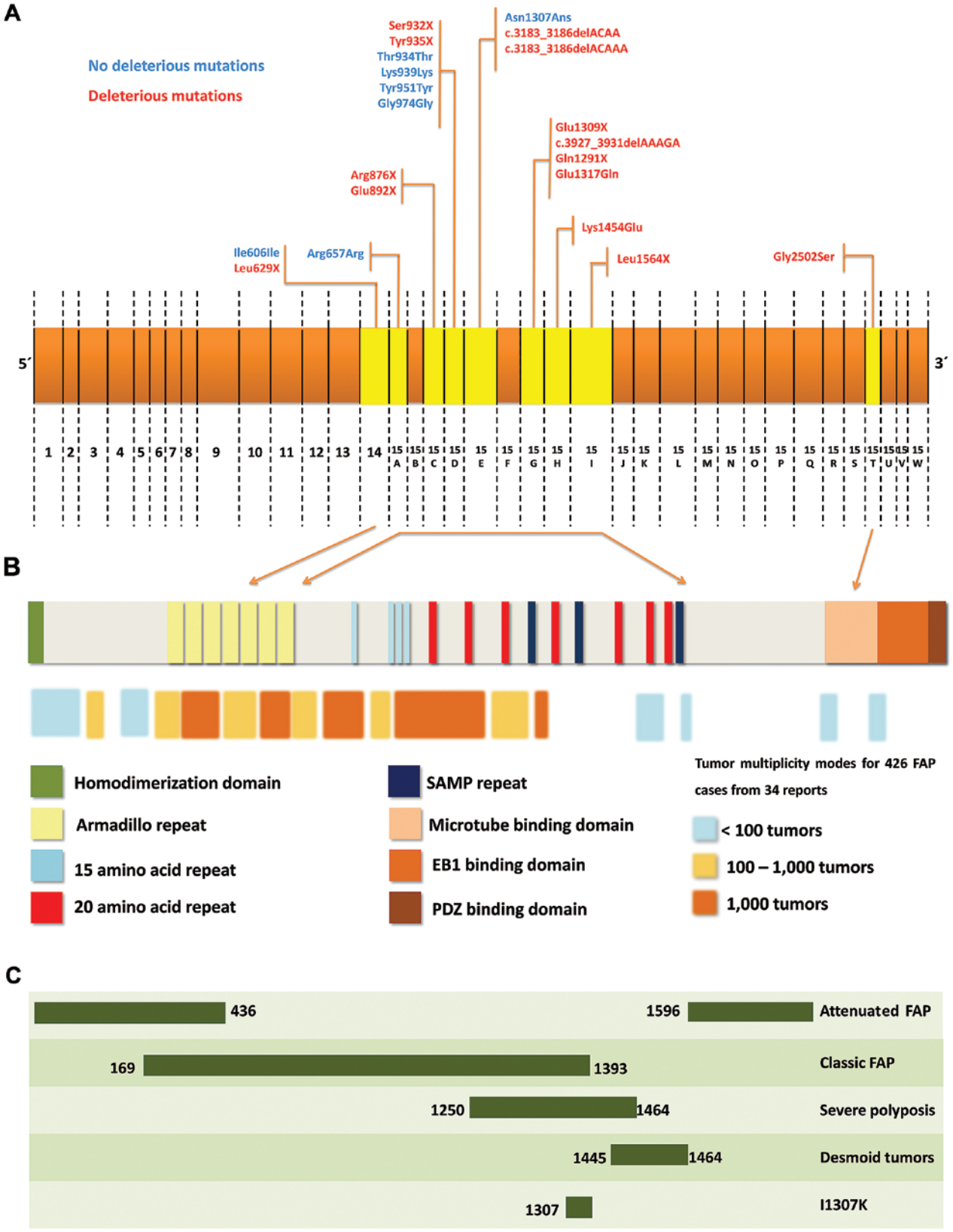

Figure 2. (A) Adenomatous polyposis coli (*APC*) germline mutation identified in the gene exon location. A deleterious mutation is shown in red; blue, no deleterious mutation is shown. All exons are shown in detail, and exons with mutations are illustrated in yellow. (B) Structure of the APC protein. The color bar below indicates the genotype-phenotype correlation of sites of protein truncation to human disease severity. (C) Genotype-phenotype correlation on the *APC* gene. FAP, familial adenomatous polyposis. Fig. 2 was adapted from previous studies (15-17).

analyzed by correlating the TNM stage with the clinical variables, and the data are shown in Table III. The same associations were analyzed between Astler-Coller stage and the clinical variables as described in the Table IV.

For the deleterious mutations detected, we found a prevalence of nonsense mutations, with 9 (45%) mutant alleles. In 4 (20%) patients small deletions were noted, while 3 (15%) patients had missense mutations, and 3 (15%) patients had only neutral polymorphisms and 1 (5%)patient had no mutations found in the exons. In 60% of patients, extra-colonic manifestations were present; the most common being gastric polyps, duodenal and in the small bowel (Table II).

In Figs. 1 and 2, the *APC* gene and all mutations identified were described in details. In the same figures, the protein structure is described, considering the principal mutation sites and their association with FAP.

Associations between the clinical variables and the identified *APC* germline mutations could not be calculated as the sample size was small and some of the mutations were not deleterious.

Discussion

The high molecular heterogeneity in the *APC* gene was consistent with other studies in FAP patients (12,18). Mutations c.3927_3931delAAAGA and pTyr935X were found in 2 patients. The c.3927_3931delAAAGA mutation occurs in exon 15 and leads to formation of a stop codon at position 1,312. It is the most frequent mutation in the *APC* gene. Its frequency varies from 0% in southwest Spain to 2.4% in the Australian population, 5% in the Dutch population, 7% in the Israeli population, and up to 16% in Italian FAP patients (19-22). The pTyr935X mutation is a nonsense alteration of exon 15 that exchanges cytosine for adenine.

In our sample, we found a predominance of nonsense mutations (45% of the patients), followed by frameshift mutations (20% of patients). Among the 6 (30%) patients with neutral mutations, missense mutations occurred in more than 1 patient. We found the missense mutation, Gly2502Ser. According to Azzopardi *et al* (18), who studied 691 patients with colorectal adenomas and 969 healthy individuals (individuals investigated for cystic fibrosis), this mutation can be found in individuals with or without adenoma, leaving a doubt as to whether this mutation is deleterious.

The mutation Glu1317Gln is described in the literature as being deleterious (23-27), although other studies considered it to be not deleterious. Azzopardi *et al* (18) found this mutation in both healthy subjects and in adenoma patients. However, we need further monitoring and analysis of these individuals with the family to gain a better understanding of this result.

For the variety of mutations, we were unable to determine a correlation between the clinical variables and the mutations detected. It is necessary to expand the sample to support such analysis. Yet, following analysis of the correlation of the presence of deleterious mutations and TNM and Astler-Coller stage, we found a positive correlation with the presence of deleterious mutations, demonstrating a more severe disease. Patients with deleterious mutations had an OR, 0.086 (IC =0.001-0.984); TNM stage I + II in comparison with III + IV, when compared with the patients with no deleterious mutations identified.

In conclusion, our study demonstrated the molecular heterogeneity of *APC* germline mutations in FAP and the difficulty in performing molecular diagnostics in a Brazilian population, since there were no mutations noted with a higher prevalence. Thus, molecular diagnostics requires further detailed evaluation, which, however is hampered by the presence of neutral mutations, and these mutations are still debatable in many populations of the world.

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